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Probing Direct Interactions between CodY and the *oppD* Promoter of *Lactococcus lactis*

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CodY of *Lactococcus lactis* MG1363 is a transcriptional regulator that represses the expression of several genes encoding proteins of the proteolytic system. These genes include *pepN*, *pepC*, *opp-pepO1*, and probably *prtPM*, *pepX*, and *pepDA2*, since the expression of the latter three genes relative to nitrogen availability is similar to that of the former. By means of in vitro DNA binding assays and DNase I footprinting techniques, we demonstrate that *L. lactis* CodY interacts directly with a region upstream of the promoter of its major target known so far, the *opp* system. Our results indicate that multiple molecules of CodY interact with this promoter and that the amount of bound CodY molecules is affected by the presence of branched-chain amino acids and not by GTP. Addition of these amino acids strongly affects the extent of the region protected by CodY in DNase I footprints. Random and site-directed mutagenesis of the upstream region of *oppD* yielded variants that were derepressed in a medium with an excess of nitrogen sources. Binding studies revealed the importance of specific bases in the promoter region required for recognition by CodY.

Genetic and biochemical research over the past decades has led to a clear picture of the proteolytic system of lactic acid bacteria. To ensure a proper nitrogen balance, several regulators are present that respond to changes in intracellular concentrations of nitrogen-containing compounds (16, 24, 28, 30). The lactic acid bacterium *Lactococcus lactis* is auxotrophic for several amino acids (6). For optimal growth in milk, it has to degrade milk proteins (e.g., α_{S1} and β- and κ-casein), because only limited amounts of free amino acids are present in this environment. An elaborate proteolytic system to release amino acids from casein, involving a number of enzymatic activities that are subject to medium-dependent regulation, has evolved in *L. lactis*. Casein degradation by *L. lactis* is a process that can be divided into three successive steps (25, 51). First, the extracellular cell wall-bound serine proteinase (PrtP) liberates peptides of various sizes from casein. In the second step, the casein-derived peptides are transported into the cell by the oligopeptide transport system (Opp) or by the di- and tripeptide transport systems (DtpP and DtpT, respectively) (45). In the last step, the internalized peptides are degraded into smaller peptides and free amino acids by a large number of cytoplasmic peptidases. Two major groups of peptidases can be discerned: the endopeptidases (e.g., PepO and PepF), which perform endolytic hydrolysis of their substrates, and the aminopeptidases (e.g., PepN, PepX, and PepC), which cleave off one or two amino acids from the free N termini of their substrates (51).

Transcription of a number of lactococcal genes encoding the proteins that constitute the proteolytic system is regulated similarly in response to peptide availability in the medium (17). Transcriptional *luxAB* fusions with the promoters of a number of peptidase, protease, and transporter genes were used to show that these genes are down-regulated in peptide-rich medium. More recently, it has been demonstrated that, at least for transcription of the oligopeptide permease genes encoded by *opp*, a homologue of the nutritional repressor CodY of *Bacillus subtilis* is responsible for this peptide-dependent regulation (18). Evidence was found that the strength of repression by *L. lactis* CodY correlated with the intracellular pool of branched-chain amino acids (BCAAs) (18, 39). These findings are supported by observations that the growth in milk of an *L. lactis* strain lacking the aminotransferases AraT and BcaT, which are involved in the catabolism of BCAAs (42, 55), is severely affected when isoleucine (Ile) or a dipeptide containing this amino acid is added. Since the growth rate of an *L. lactis codY* mutant was not altered by addition of Ile, inhibition by this amino acid is probably due to CodY-mediated repression of the proteolytic system, which leads to retarded growth (5, 36). In fact, it has been shown recently that in the gram-positive bacterium *B. subtilis*, BCAAs directly interact with CodY and enhance the affinity for its targets (48). CodY was first identified in this organism, where it serves as a nutritional repressor of the dipeptide permease operon (47, 49) and of genes involved in amino acid metabolism (7, 12, 13, 54), carbon and energy metabolism (23), motility (1), antibiotic production (21), and competence development (38, 46). In addition, the affinity of *B. subtilis* CodY for its targets is stimulated by its interaction with the cofactor GTP, independently of that with the BCAAs (1, 23, 41, 48). The regulator is thereby thought to sense both the energy state and the nitrogen availability of the cell. In contrast, recent observations imply that lactococcal CodY probably does not respond to GTP, since addition of

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype or genotype	Source or reference
Strains		
Lactococcus lactis subsp. *cremoris*		
MG1363	Lac$^-$ Prt$^-$; plasmid-free derivative of NCDO712	15
LL108	Cm^r; MG1363 derivative containing multiple copies of the pWV01 *repA* gene in the chromosome	32
LL302	RepA$^+$ MG1363, carrying one copy of the pWV01 *repA* gene in the chromosome	32
NZ9000	MG1363 *pepN*::*nisRK*	27
NZ9700	Nisin-producing transconjugant of MG1363 containing the nisin-sucrose transposon Tn*5276*	26
E. coli EC101	Km^r; JM101 with *repA* from pWV01 integrated into the chromosome	31
Plasmids		
pNZ8048	Cm^r; inducible expression vector carrying P_{nisA}	27
pNH6CodY	*his6-codY* of *L. lactis* MG1363 behind P_{nisA}	This work
pORI13	Em^r; integration vector; promoterless *lacZ*; Ori$^+$ RepA derivative of pWV01	44
pORIopp68	Em^r; pORI13 carrying a 160-bp *oppD* promoter fragment amplified with primers opp1 and opp2	This work
pORIopp111	Em^r; pORI13 carrying a 203-bp *oppD* promoter fragment amplified with primers opp1 and opp11	This work
pORIopp162	Em^r; pORI13 carrying a 254-bp *oppD* promoter fragment amplified with primers opp1 and opp3	This work
pORIopp14	Em^r; pORI13 carrying a 170-bp *oppD* promoter fragment amplified with primers opp1 and opp14	This work
pORIopp15(a)	Em^r; pORI13 carrying a 170-bp *oppD* promoter fragment amplified with primers opp1 and opp15(a)	This work
pORIopp15(b)	Em^r; pORI13 carrying a 170-bp *oppD* promoter fragment amplified with primers opp1 and opp15(b)	This work

decoyinine to the medium, which evokes a rapid drop in intracellular GTP levels, did not result in derepression of a CodY target gene (39).

CodY contains a C-terminal helix-turn-helix motif, and the *B. subtilus* protein has been shown to bind to sequences overlapping the −35 and −10 sequences of its target promoters (13, 47). Although the binding of CodY to several targets has been demonstrated, no consensus recognition sequence for CodY binding has been deduced (12). The present study aims to improve our understanding of *L. lactis* CodY by studying its binding to one of its DNA targets, the *opp* region. In order to examine whether repression by CodY occurs by direct protein-DNA interactions, DNA binding and DNase I footprinting studies were performed. By combining a random and a site-directed mutagenesis approach, we show the importance of several nucleotides in the promoter region of *opp* for recognition by CodY.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown in tryptone-yeast extract (TY) medium (Difco Laboratories, West Molesey, United Kingdom) at 37°C with vigorous agitation or on TY medium (2) solidified with 1.5% agar, containing 100 μg of erythromycin per ml where needed. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used at a final concentration of 40 μg/ml. *L. lactis* was grown at 30°C in 0.5× M17 broth (50) or on 0.5× M17 medium solidified with 1.5% agar and supplemented with 0.5% glucose (GM17). When appropriate, erythromycin, chloramphenicol, and X-Gal were added at final concentrations of 5, 5, and 80 μg per ml, respectively. Antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.). A chemically defined medium (CDM) was prepared as described previously (29) and supplemented with Casitone (Difco Laboratories) as a nitrogen source where indicated.

DNA preparation, molecular cloning, and transformation. Routine DNA manipulations were performed as described elsewhere (43). Total chromosomal DNA from *L. lactis* MG1363 was extracted as described previously (33). Plasmid DNA was isolated by the alkaline lysis procedure as described elsewhere (43). Minipreparations of plasmid DNA from *E. coli* and *L. lactis* were essentially made by using the High Pure plasmid isolation kit from Roche Molecular Biochemicals (Mannheim, Germany). Restriction enzymes and T4 DNA ligase were purchased from Roche Molecular Biochemicals. PCR amplifications were carried out using either Pwo DNA polymerase for cloning purposes or *Taq* DNA polymerase (both from Roche Molecular Biochemicals) for checking plasmid DNA insert sizes from transformants. PCR products were purified with the High Pure PCR product purification kit (Roche Molecular Biochemicals). Electrotransformations of *E. coli* and *L. lactis* were performed using a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) as described previously (9, 20).

RNA preparation and primer extension. The *opp* transcript was subjected to primer extension analysis using oligonucleotide sto14 (see Table 2) essentially as described previously (4). In the reactions, 30 μg of total RNA that was isolated from *L. lactis* MG1363 cells as described previously (52) was used as a template. A DNA sequence ladder was obtained by using the T7 sequencing kit (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom) according to the manufacturer's descriptions.

Cloning of *oppD* promoter fragments. Oligonucleotides used to amplify *oppD* promoter fragments are listed in Table 2. Combinations of oligonucleotide opp1 with oligonucleotides opp2, opp3, opp11, opp14, opp15(a), and opp15(b) were used to amplify chromosomal DNA from *L. lactis* MG1363, in order to obtain various fragments encompassing the *oppD* promoter. The PCR products were digested with XbaI and PstI and were transcriptionally fused upstream of the promoterless *lacZ* gene in the pORI13 integration vector (44) digested with the same enzymes. The resulting plasmids, pORIopp68, pORIopp162, pORIopp111, pORIopp14, pORIopp15(a), and pORIopp15(b), respectively, were all isolated by using *E. coli* EC101, which contains a chromosomal copy of the lactococcal *repA* gene, needed for pORI13 replication, as the cloning host. After isolation, the plasmids were introduced by electroporation into *L. lactis* LL302 and/or *L. lactis* LL108, which contain single and multiple chromosomal copies of *repA*, respectively, to facilitate replication of the pORI13 derivatives (32).

TABLE 2. Oligonucleotides used in this study

Name	Sequence (5′→3′)[a]
hc-5	CTAGACCACCATGGGG*CATCACCATCACCATCAC*GTGGCTACATTACTTGAAAAAACACG
hc-6	CTAGTCTAGA*TTA*GAAATTACGTCCAGCAAGTTTATC
opp1	GCTCTAGACACTCACTTGTTTTGCTTCC
opp2	AACTGCAGGAAAATTCATGAACATACC
opp3	AACTGCAGTAAAACAATAATAAAAGCAG
opp11	AACTGCAGCTCCAAAACTTTTGCTTTAC
opp14	AACTGCAGCGTAATGTTCAGAAAATTC
opp15(a)	AACTGCAGCGTAATATTTAGAAAATTCATGAACATACC
opp15(b)	AACTGCAGCGTACTGTGCCGAAAATTCATGAACATACC
sto14	CTTGCCATGGAATCACCCG

[a] Restriction enzyme sites are underlined. Italicized sequence in hc-5 encodes the hexahistidine tag. Italicized sequence in hc-6 is the stop codon.

Random mutagenesis of the *oppD* promoter region. PCR fragments encompassing the *oppD* promoter region containing random base pair substitutions were obtained by using the Diversify PCR random mutagenesis kit (Clontech Laboratories, San Jose, Calif.). *L. lactis* MG1363 chromosomal DNA was used as a template in the amplification step. Subsequently, the variants obtained were cloned into plasmid pORI13 and introduced into *L. lactis* LL108 as described above. Mutants showing differential blue coloring (compared to the unmutated *oppD* region cloned into pORI13) on GM17 plates containing X-Gal were selected and analyzed in more detail as described below.

Determination of β-galactosidase activity in *L. lactis*. Overnight cultures of *L. lactis* grown in GM17 were washed twice in 0.9% NaCl before inoculation to 1% in 50 ml of the appropriate medium containing erythromycin for the maintenance of pORI13 in *L. lactis* LL108 or LL302. Exponential-phase cells (optical density at 600 nm, ~1.0) were collected by centrifugation. β-Galactosidase activities were determined in permeabilized cell suspensions as described previously (22). β-Galactosidase enzyme activities, calculated as averages from three independent experiments, were expressed in arbitrary units (37).

Purification of histidine-tagged CodY. The *codY* gene of *L. lactis* MG1363 was amplified from the chromosome by PCR with oligonucleotides hc-5, introducing the NcoI restriction enzyme site upstream of the sequence encoding the hexahistidine tag, and hc-6, introducing the XbaI restriction enzyme site downstream of the stop codon of the resulting His-tagged *codY*, designated *H6-codY*. The purified 833-bp PCR product was digested with NcoI and XbaI and ligated into the corresponding sites in pNZ8048, resulting in pNH6CodY. This plasmid was introduced into *L. lactis* NZ9000 to enable nisin-induced expression from P*nisA* upstream of *H6-codY*, as described previously (8, 27). Following induction, H6-CodY was isolated from lysates of induced cells by affinity chromatography in a procedure involving fast-performance protein liquid chromatography (Amersham Pharmacia Biotech) using nickel-nitrilotriacetic acid agarose (QIAGEN GmbH, Hilden, Germany). The concentration of the purified protein was determined by the Bradford procedure (3).

Electrophoretic mobility shift assays (EMSAs). Gel retardation experiments were carried out essentially as described elsewhere (10). Purified PCR products (2 μg) were end labeled with polynucleotide kinase (Amersham Pharmacia Biotech) for 1 h at 37°C by using 30 μCi of [γ-^{32}P]ATP (Amersham Pharmacia Biotech) in a volume of 20 μl. Reactions were stopped by incubating the mixtures for 10 min at 70°C. Binding studies were carried out in 20-μl reaction volumes containing 20 mM Tris-HCl (pH 8.0), 8.7% (vol/vol) glycerol, 1 mM EDTA (pH 8.0), 5 mM $MgCl_2$, 100 mM KCl, 0.5 mM dithiothreitol, labeled DNA fragment (3,000 cpm), and purified H6-CodY protein (50 to 400 ng). Bovine serum albumin (1 μg) and poly(dI-dC) (Amersham Pharmacia Biotech) were added to the reaction mixtures in order to reduce nonspecific interactions. After incubation for 15 min at 30°C, samples were loaded onto a 4% polyacrylamide gel. Electrophoresis was performed in the Protean II Minigel system (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands) by using a gradient (0.5× to 2×) of Tris-acetate-EDTA buffer (43) at 150 V for 1.5 h. Gels were dried and used for autoradiography at −80°C by using Kodak XAR-5 film and intensifying screens.

DNase I footprinting analysis. DNase I footprinting was performed essentially according to the description supplied with the Sure Track footprinting kit (Amersham Pharmacia Biotech). The DNA fragments were prepared by PCR using Expand DNA polymerase (Amersham Pharmacia Biotech) and oligonucleotides opp1 and opp3, one of which was first end labeled with T4 polynucleotide kinase (Amersham Pharmacia Biotech) and [γ-^{32}P]ATP as described by the manufacturer. Binding reactions were identical to those used in EMSAs, in a total volume of 40 μl and in the presence of approximately 150,000 cpm of the DNA probe. Subsequently, DNase I footprinting experiments were performed as described previously (19).

RESULTS

Determination of the TSS of *oppD*. It has been demonstrated that the genes of the oligopeptide permease system, carried on the *oppDFBCA-pepO1* locus of *L. lactis* MG1363, are transcribed polycistronically (17, 51). Upstream of both *oppD* and *oppA* are regions that could serve as promoter elements. To precisely determine the location of the promoter upstream of *oppD*, the transcription start site (TSS) was determined. The *opp* transcript was analyzed by primer extension using RNA that was isolated from exponentially growing MG1363 cells (Fig. 1A). The mRNA 5′ end corresponds to an adenine residue located 35 bases upstream of the translation start codon (AUG) of *oppD*. The −35 sequence (TTGCAA) is separated by a consensus 17 bp from the −10 region (TATACT), and a proper lactococcal ribosome binding site (RBS), with the sequence GAGG, is also present (Fig. 1B). Although the distance between the −10 region and the TSS is several base pairs shorter than those for most other lactococcal promoters (53), efficient transcription initiation is allowed, as evidenced by the fact that expression from P*oppD* was readily detectable (see Fig. 2D). The sequence upstream of *oppD* contains two regions of dyad symmetry centered on positions −135 (−14.0 kcal/mol) and −62 (−5.6 kcal/mol) relative to the *oppD* TSS, respectively.

H6-CodY interacts with the region upstream of *oppD*. In order to examine whether *L. lactis* CodY directly interacts with its main target known so far, the *opp-pepO1* region, in vitro DNA binding studies were performed using probes spanning P*oppD*. For this purpose, CodY carrying a histidine tag at its N terminus (H6-CodY) was overexpressed in *L. lactis* by using the nisin-inducible gene expression system (8) and was subsequently purified to apparent homogeneity (data not shown). A 254-bp radioactively labeled DNA probe (*opp162*), spanning the *oppD* promoter from position −162 to +75 relative to the TSS, was prepared by PCR as described in Materials and Methods (Fig. 2A). EMSAs clearly showed that purified H6-CodY was capable of binding directly to this fragment (Fig. 2B). Multiple retarded fragments were present, indicating that several molecules of H6-CodY can bind to the *oppD* fragment. When the amount of H6-CodY was gradually increased from 0 to almost 1,000 nM, at least three distinct bands could be

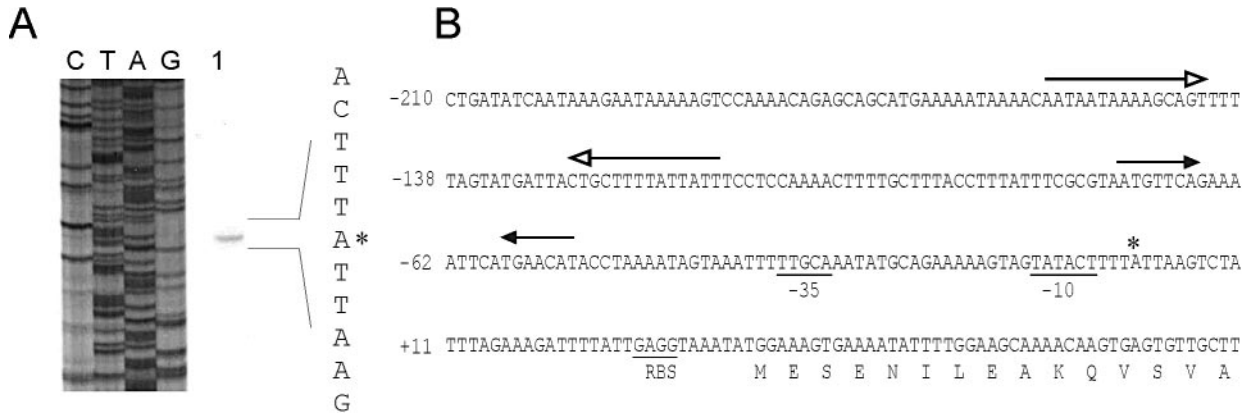

FIG. 1. Overview of the region upstream of *oppD* of *L. lactis* MG1363. (A) Primer extension analysis of P*oppD*. Primer extension reactions were performed with 30 μg of RNA isolated from exponentially growing *L. lactis* MG1363 in GM17 and primer sto14. The sequencing ladder (CTAG) and the extension reaction product (lane 1) are shown. The position of the transcription start site, in a part of the sequence given in the right margin, is marked by an asterisk. (B) Detailed view of the region upstream of *oppD*. The −35, −10, and RBS sequences are underlined. The positions of two regions of dyad symmetry are indicated by arrows with open or closed arrowheads. The position of the transcription start site is marked by an asterisk.

discerned, possibly reflecting a multimeric state of the protein or binding of CodY to multiple independent binding sites. In most of our DNA binding experiments, a band corresponding to the single-stranded probe (Fig. 2B) was observed irrespective of the presence of H6-CodY. The occurrence of this denatured DNA probably resulted from the high AT content of the *opp* promoter region. No H6-CodY binding occurred when DNA fragments with similar AT contents that were obtained from internal gene segments (e.g., from *comG* of *B. subtilis*) were used, indicating that H6-CodY binding to P*oppD* is specific (data not shown).

To get a better indication of which part of the region upstream of *oppD* is important for interaction with CodY, deletion analysis was performed. Two truncated fragments, shortened from the 5′ end of the *oppD* upstream region contained in *opp162*, were obtained by PCR and examined for H6-CodY binding (Fig. 2A and C). Probe *opp111*, spanning the region from −111 to + 75, was bound by H6-CodY similarly to *opp162* (data not shown), suggesting that nucleotides that are critical for CodY binding must reside downstream of the 5′ end of this probe. When a 160-bp DNA fragment (probe *opp68*), the 5′ end of which coincides with the center of the inverted repeat (IR) closest to the RBS of *oppD* (Fig. 1A), was used, binding of H6-CodY was almost completely abolished, since the probe would shift only at high concentrations of protein and labeled DNA, and fewer molecules of H6-CodY appeared to bind (Fig. 2A and C).

To verify that the in vitro binding of the three probes by CodY reflected the in vivo situation, the fragments were fused upstream of the promoterless *lacZ* gene in plasmid pORI13 (44) and introduced into *L. lactis* LL108. Subsequently, β-galactosidase activities in cells growing exponentially in media differing in their peptide contents were determined. In CDM supplemented with 2% Casitone, where strong CodY-mediated repression is ensured (18), expression driven from promoter constructs derived from the two longer probes (*opp162* and *opp111*) seemed to be strongly repressed, in contrast to the behavior of the strain carrying the *opp68*-derived reporter fusion, which displayed approximately 30- to 40-fold-higher expression. In CDM containing 0.2% Casitone, where CodY-mediated repression is strongly relieved, expression from the *opp162* and *opp111* fragments was derepressed about ninefold whereas regulation of the *opp68* fragment was much lower. Thus, the strength of regulation of P*oppD*-driven expression seemed to correlate with the observed binding pattern (Fig. 2D), suggesting that at least part of an operator site for CodY must be located in a region between positions −111 and −68 relative to the *oppD* TSS.

H6-CodY protects an extended region of the *oppD* promoter. Although the EMSA experiments using truncated P*oppD* DNA fragments pointed to a specific region that is important for CodY binding, the actual area facilitating binding was rather large (positions −68 to −111). Moreover, the assays did not exclude the possibility that more downstream sequences could contribute to CodY binding. Therefore, formation of a complex by H6-CodY and the *oppD* promoter region was investigated by DNase I footprinting experiments using the labeled promoter fragment *opp162* and the binding conditions used for the EMSAs (Fig. 3). H6-CodY binding resulted in the protection of bases extending from position −80 to −20 and position −80 to −10 relative to the TSS of the upper and lower strands, respectively. In addition to these protected regions, both DNA strands contained hypersensitive sites when they were incubated with DNase I in the presence of H6-CodY. These results show that H6-CodY binds to a region encompassing the −35 to −10 sequences of the promoter of *oppD*.

A region in the *oppD* promoter containing an IR is important for CodY-mediated regulation. A closer inspection of the area in the *oppD* promoter to which CodY binds revealed the presence of a short sequence (ATGTTCA) that is inversely repeated, with a spacing of 9 bp between the partners (Fig. 1). This region of dyad symmetry is located 18 bp upstream of the −35 sequence and is entirely present in probes *opp162* and *opp111*, to which H6-CodY binds (Fig. 2A and B). The fact that H6-CodY was hardly able to form a protein-DNA complex when a probe lacking the upstream arm of this IR (probe

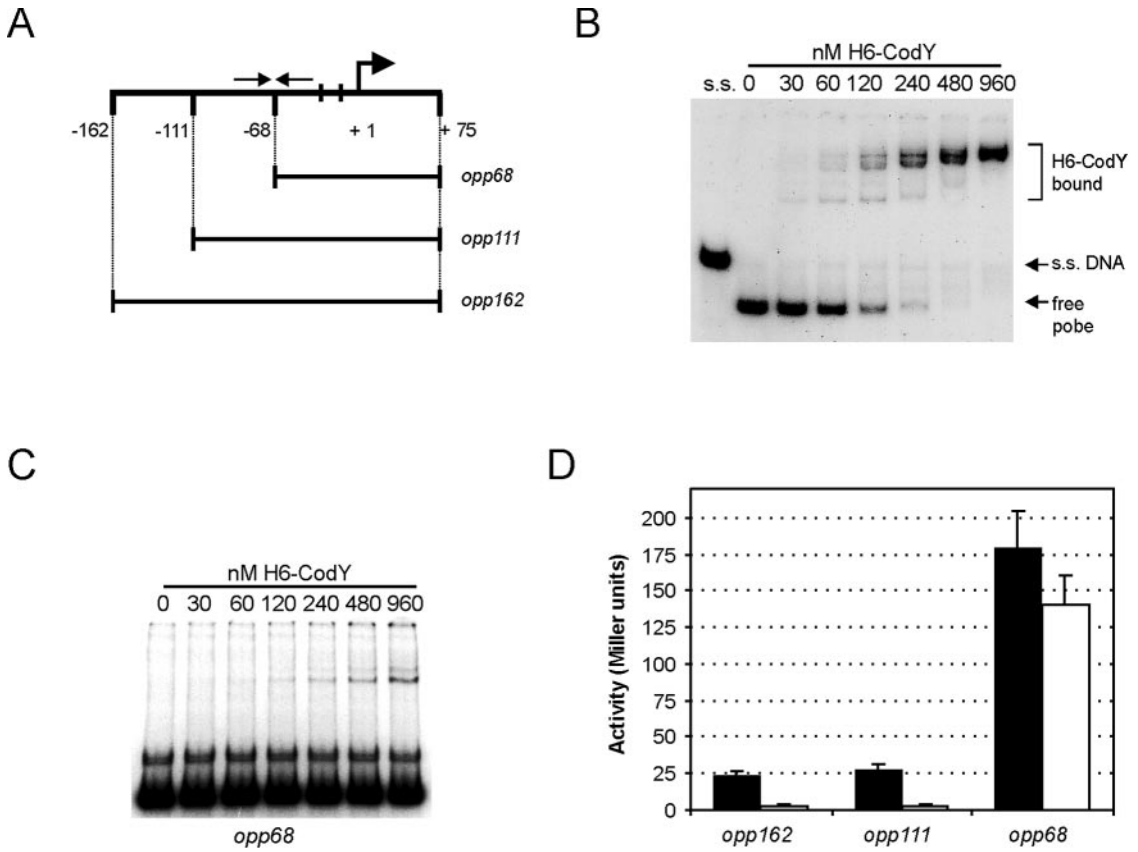

FIG. 2. Interaction of H6-CodY with fragments of the *oppD* promoter region. (A) Schematic view of the probes used in panels B and C. Probes *opp162*, *opp111*, and *opp68* were obtained by PCR on chromosomal DNA of *L. lactis* MG1363, using a combination of oligonucleotide opp1 with oligonucleotide opp3, opp11, or opp2, respectively. Arrows indicate the position of the region of dyad symmetry closest to the −35 sequence (see Fig. 1B). Vertical bars indicate the positions of the −35 and −10 sequences. Nucleotide positions are relative to the transcriptional start site (right-turn arrow at +1). (B) Interaction of H6-CodY with the upstream region of *oppD*. The radioactively labeled probe *opp162* was incubated alone or with increasing amounts of purified H6-CodY. The first lane contains the same probe, which was boiled in a 95% formamide solution in order to obtain single-stranded (s.s.) DNA fragments. (C) H6-CodY binding to probe *opp68*. The same conditions were used as for probe *opp162* (B). (D) In vivo activities of P*oppD* variants. *L. lactis* LL108 strains carrying *lacZ* reporter plasmids fused to the *opp* fragments depicted schematically in panel A were grown in CDM containing either 0.2 or 2% Casitone (solid and open bars, respectively). Cells were harvested in the exponential phase of growth, and β-galactosidase activity was measured. Assays were carried out twice, in triplicate each time. Error bars, standard deviations.

opp68 [Fig. 2A and C]) was used implies that this area might serve as an operator site for CodY on P*oppD*. To study this region in more detail, site-directed mutations were introduced by PCR using combinations of oligonucleotide opp1 with oligonucleotide opp14 (wild type), opp15(a), opp(15b), or opp2 (Fig. 4A). The PCR products were inserted upstream of the promoterless *lacZ* reporter gene in pORI13 and introduced into *L. lactis* LL108. Introducing base substitutions into the upstream arm of the repeat [*opp15*(*a*) and *opp15*(*b*)] resulted in both weaker binding of H6-CodY (Fig. 4B) and derepression of P*oppD*-driven *lacZ* expression (Fig. 4C). When the unchanged half-site was replaced by an unrelated sequence (an XbaI endonuclease site, present in *opp2*), repression was reduced approximately 30-fold (in cells growing exponentially in a rich medium) and H6-CodY binding was almost completely abolished (as shown in Fig. 2C). Converting both the C and G residues in this region to adenines [*opp15*(*a*)] resulted in a ~7-fold reduction of repression, whereas changing 3 out of 6 bases [*opp15*(*b*)] led to a >20-fold derepression of expression. These results were in accordance with those obtained from gel retardation analyses (Fig. 4B), where the affinity of H6-CodY was highest for the wild-type probe, intermediate for the *opp15*(*a*) probe, and lowest for the *opp15*(*b*) probe. In the case of the *opp15*(*b*) probe, hardly any protein-DNA complexes were present and all intermediate complexes that were observed with the wild-type and *opp15*(*a*) fragments were absent.

Random mutation analysis of the *oppD* promoter area. As shown above, a region close to the −35 sequence seems to be important for the binding of CodY to the *oppD* regulatory region. Since we could not find any obviously similar sequences in the upstream regions of any of the other CodY-regulated genes identified so far, it is possible that structural determinants are required for CodY recognition of a promoter region. Therefore, PCR-based random mutagenesis was carried out on the smallest *oppD* promoter fragment still showing strong repression of transcription by CodY (probe *opp111* [Fig. 2]). PCR fragments containing random base pair substitutions or small deletions were restricted by using the appropriate restric-

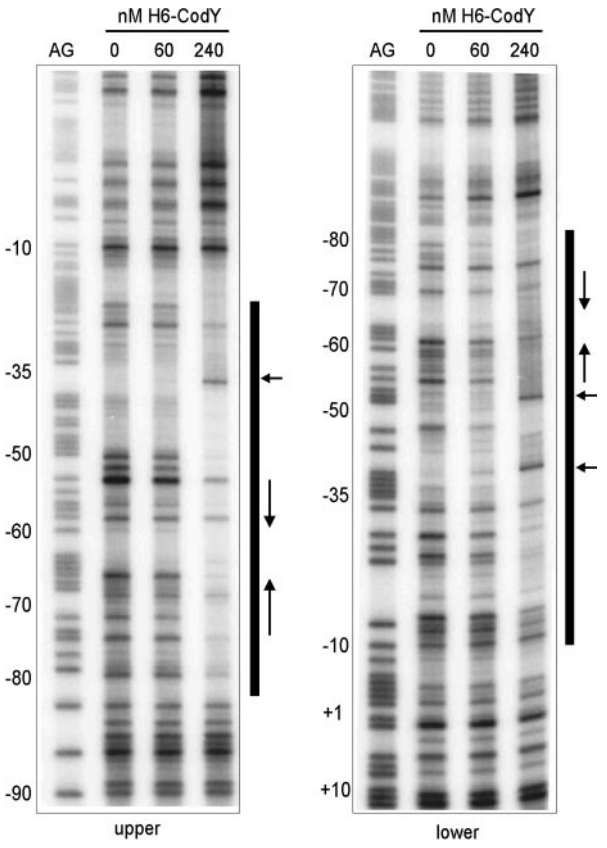

FIG. 3. DNase I footprinting analysis of H6-CodY binding to the *oppD* promoter region. The left and right panels show the footprints of the upper and lower strands, respectively. Footprints, obtained in the absence or presence of 60 or 240 nM H6-CodY by using radioactively labeled probe *opp162*, are flanked by Maxam and Gilbert A+G sequence ladders (AG) on the left. Numbers on the left indicate base pair positions relative to the TSS. Protected regions are marked with bars, and horizontal arrows indicate the positions of hypersensitive bonds. Vertical arrows indicate the region of dyad symmetry closest to the −35 sequence (see Fig. 1B).

tion enzymes and ligated upstream of the promoterless *lacZ* in pORI13. The ligation mixture was used to transform *L. lactis* LL108. Cells carrying a pORI13 construct with a P*oppD* fragment containing a derepressing mutation formed light-blue or blue colonies on CDM plates containing X-Gal and excess nitrogen sources (in the form of Casitone), as opposed to the whitish color of colonies formed by cells harboring pORI13 with wild-type P*oppD*. The *oppD* fragments in cells with a derepressed phenotype were amplified by PCR, radioactively labeled, and tested by an EMSA for their abilities to form complexes with H6-CodY. The relative strength of H6-CodY binding was examined by comparing the amount of the retarded mutated DNA fragment resulting from H6-CodY binding to that of the unchanged P*oppD* fragment (Fig. 5). Weaker binding to all of the mutated promoter fragments was observed. Sequence analyses of the P*oppD* variants obtained in this study revealed that all of them carried one or more base pair substitutions, at least one of which was located in the region from −82 to −56 relative to the TSS of *oppD*. This finding, again, is an indication of the importance of this region for CodY binding.

BCAAs stimulate stimulate H6-CodY binding. Evidence has been presented that CodY senses the nitrogen supply of the cell as a function of the BCAA pool (18, 39). Although BCAAs act as direct effectors of CodY activity in *B. subtilis* (48), the exact nature of this signal in *L. lactis* remains to be established. Therefore, in vitro DNA binding of H6-CodY was examined in the presence or absence of the three BCAAs Val, Leu, and Ile (Fig. 6A). Addition of any of the three BCAAs resulted in increased binding of H6-CodY to the P*oppD* probe *opp162* compared to a situation in which no amino acid or another, aliphatic amino acid (i.e., alanine) was present. Stimulation of H6-CodY binding by Val and Leu was observed at concentrations of these amino acids above 10 mM. In a titration of Ile and a constant amount of H6-CodY, most of the probe DNA was already retarded in the presence of 5 mM Ile, showing that this BCAA stimulates the binding of H6-CodY to the highest extent.

The role of BCAAs in CodY binding was also investigated by means of DNase I footprinting experiments, in which the *opp162* probe was incubated with H6-CodY either alone; in the presence of 20 mM Val, Leu, or Ile; or with a combination of these three BCAAs (Fig. 6B). Addition of any of these BCAAs resulted in extended protection of P*oppD*. As in the EMSAs, the effect of Ile addition was most severe. These results show that the binding of lactococcal CodY to the target region of *oppD* is directly stimulated by BCAAs and that, as in *B. subtilis*, Ile is most effective.

Similarly, we also tested whether GTP could stimulate the binding of H6-CodY to lactococcal P*oppD*, since GTP serves as an effector molecule that enhances the binding of *B. subtilis* CodY to a number of its targets. As can be seen from Fig. 6C, the binding of *L. lactis* H6-CodY was not affected by the presence of GTP at a range of concentrations between 0.25 and 2.0 mM. These results are in good agreement with recent evidence showing that lactococcal CodY activity is independent of GTP at physiological concentrations, which do not exceed 0.55 mM in a medium containing Casitone (39).

DISCUSSION

L. lactis possesses a number of genes whose products are involved in the utilization of proteins present in milk, such as an extracellular protease, peptide transport systems, and intracellular peptidases (24). Most of these genes have been cloned and sequenced, and their enzymes have been biochemically characterized (24). Recently, it has been established that CodY plays a pleiotropic role in the regulation of a number of these genes in response to nitrogen availability (17, 18).

The present study was conducted to gain insight into the role of CodY in the regulation of the *opp* system and, more specifically, into the molecular interactions between the *cis* site and CodY. By combining data from in vivo and in vitro experiments, we have clearly demonstrated that repression by CodY is mediated by direct binding of this protein to the *oppD* promoter region. For this to occur most efficiently, binding of several molecules of CodY is probably required, since several protein-DNA complexes were discerned in all EMSAs in which variants (in size or base composition) of this promoter frag-

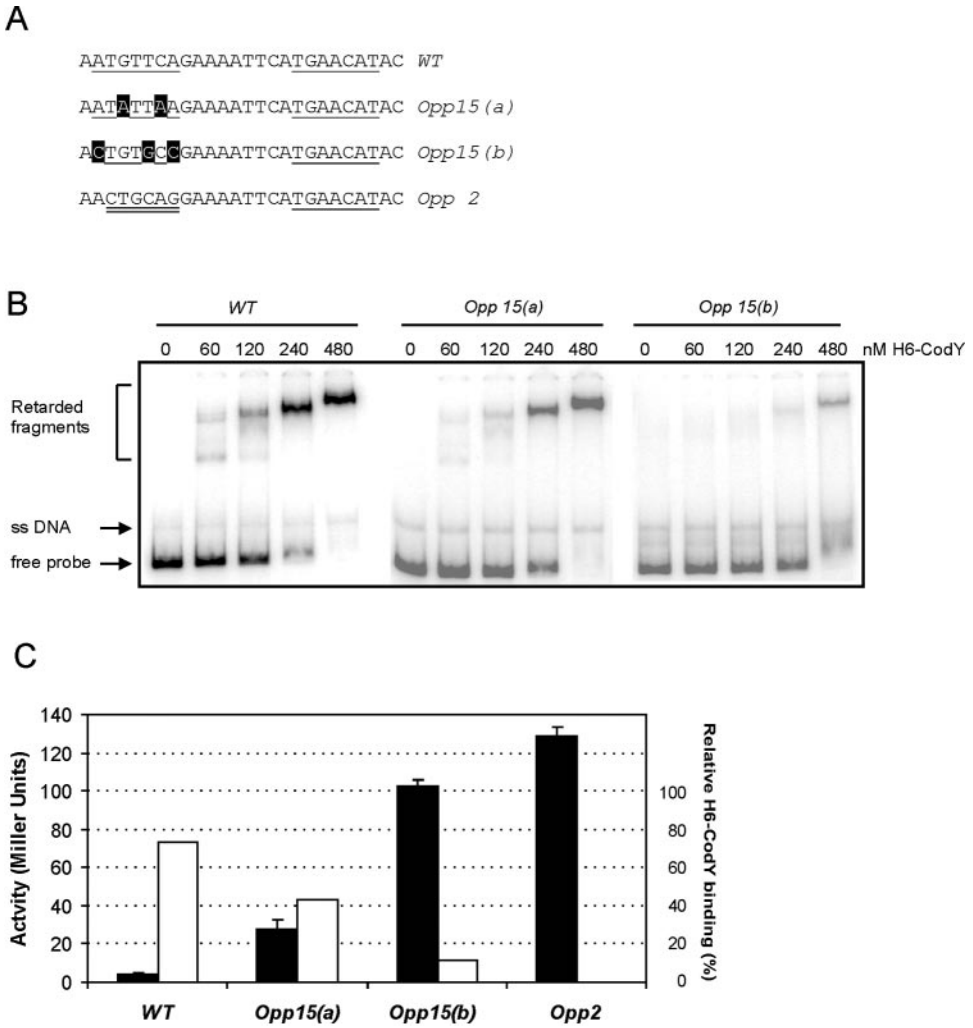

FIG. 4. Site-directed mutagenesis of the region of dyad symmetry closest to the RBS in the *oppD* promoter region. (A) Three variants of P*oppD* that contain mutations in the left arm of the IR (underlined) were obtained by PCR and cloned upstream of *lacZ* in pORI13 as described in Materials and Methods. The *WT* fragment contains no substitutions; variants *opp15*(*a*) and *opp15*(*b*) contain 2- and 3-bp substitutions, respectively (highlighted). In *opp2* an XbaI endonuclease site (double underlined) preceded by two adenine residues replaced the left arm. (B) EMSA using H6-CodY and the indicated radioactively labeled P*oppD* variants. Binding reactions were performed as described in the legend to Fig. 2. (C) Promoter activities of P*oppD* variants. *L. lactis* LL108 strains carrying the *lacZ* reporter plasmids were grown in GM17. Cells were harvested in the exponential phase of growth, and β-galactosidase activity was measured (solid bars). The experiments were carried out in triplicate. Error bars, standard deviations. Open bars, quantitative representation of the DNA binding assay for which results are shown in panel B; the relative binding affinities of H6-CodY for the P*oppD* variants were calculated by comparing the intensity of the shifted, H6-CodY bound complexes with the total radioactive signal in each lane in the presence of 240 nM protein.

ment were retarded by H6-CodY. Formation of multiple retarded DNA fragments was also observed in EMSAs using *B. subtilis* CodY with promoter DNA fragments of the *B. subtilis* dipeptide permease operon, *dpp* (47). Similar results were obtained in EMSAs that we performed using lactococcal H6-CodY with probes encompassing upstream regions of the peptidase genes *pepN* and *pepC* (data not shown) and an intergenic region containing the divergently transcribed promoters of *prtP* and *prtM* (14), which encode the proteinase and proteinase maturase of *L. lactis*, respectively (34, 35).

The observations that several molecules of CodY are able to interact with target DNA (Fig. 2), together with the DNase I footprinting data (Fig. 3) showing that a region encompassing the −35 area of the promoter is protected, lead to a tentative model in which CodY binding is thought to prevent access of RNA polymerase to the promoter, thereby hampering transcription initiation. DNA binding studies using reconstituted lactococcal RNA polymerase would help to elucidate such a mechanism.

As mentioned, the intracellular pool of BCAAs exerts an influence on the activity of CodY in *L. lactis*, but the exact nature of this signal remains to be elucidated (18). In addition, as was also demonstrated for *B. subtilis* CodY, lactococcal CodY appears to bind quite well to its targets in the absence of any cellular components in in vitro DNA binding studies, although we cannot fully exclude the possibility that cofactors were copurified with H6-CodY. Here we show that BCAAs alone could function as cofactors for CodY activity, since the

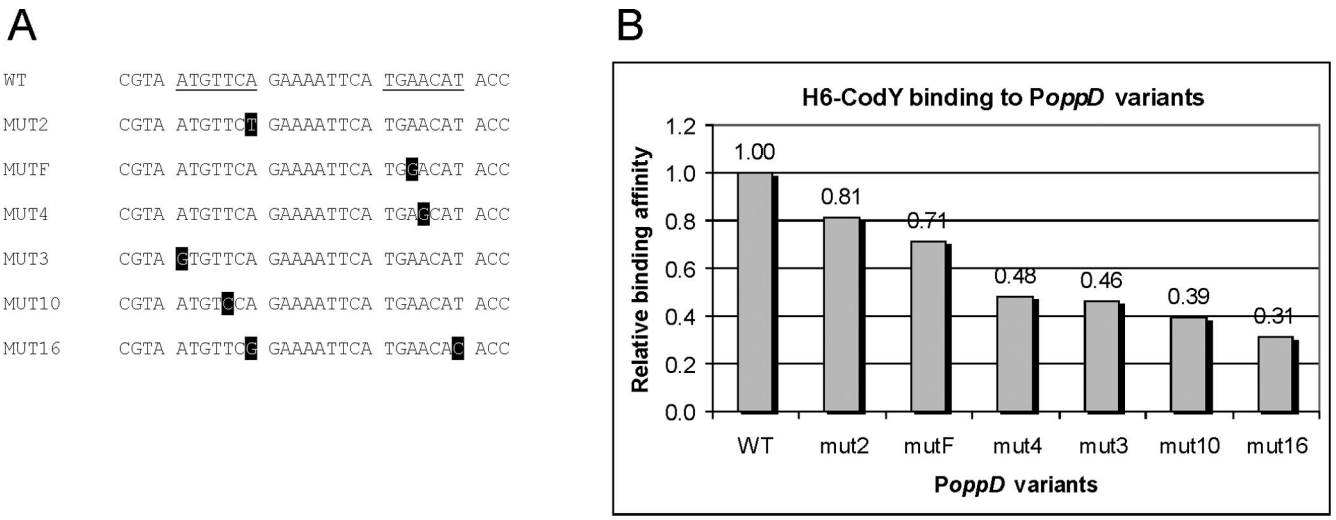

FIG. 5. H6-CodY binding to derepressed variants of P*oppD*. (A) Positions in P*oppD* of the base pair substitutions (highlighted) that led to distorted repression by CodY relative to the wild-type (WT) fragment. Underlining marks the inverted repeat closest to the −35 sequence of P*oppD*. (B) Binding of H6-CodY to labeled PCR products encompassing the mutated promoter regions presented in panel A in an in vitro binding assay, relative to its binding to the wild-type P*oppD* fragment. The relative affinity was calculated by comparing the amount of H6-CodY required to shift 50% of the labeled DNA in the binding assay.

binding properties of H6-CodY are greatly altered by the addition of any of these amino acids (Fig. 6). Ile in particular strongly enhances the binding of H6-CodY to P*oppD*, which suggests that the intracellular pool of this amino acid is important for the modulation of CodY activity. Recently, intracellular concentrations of BCAAs have been determined in exponentially growing cells (40). It was shown that when Casitone was added to the growth medium, the BCAA concentration increased to almost 10 mM and CodY-mediated repression occurred. These data are consistent with our DNA binding assays, which show that CodY binding is stimulated by BCAAs at this concentration (Fig. 6) and support the view that these amino acids might directly activate lactococcal CodY (39). Thus, *B. subtilis* and *L. lactis* respond similarly to intracellular BCAA levels.

Whereas GTP, which is a marker of the energy state of the cell, has a modulating effect in *B. subtilis* on the activity of CodY by enhancing its affinity for its targets (1, 23, 38, 41), such a stimulatory effect on the *L. lactis* repressor seems to be absent. In our in vitro DNA binding experiments, using probes encompassing P*oppD*, addition of GTP did not enhance binding of CodY (Fig. 6), a result in agreement with the observation that a drop in the intracellular GTP level does not result in derepression of a CodY target gene (39). These results support the possibility that *L. lactis* CodY does not sense the energy state of the cell, unlike its *B. subtilis* counterpart. It is tempting to speculate that this would explain why, thus far, the *L. lactis* CodY regulon seems to comprise only genes involved in nitrogen metabolism, while *B. subtilis* CodY appears to serve as a factor that couples nitrogen to carbon metabolism. However, DNA binding experiments using promoter DNA fragments of other members of the lactococcal CodY regulon will have to be performed, since it is possible that, as in *B. subtilis*, GTP does stimulate *L. lactis* CodY binding to some of its other targets (38).

Although several CodY-regulated genes of *B. subtilis* and *L. lactis* have been described, a consensus binding site, if any, remains to be elucidated for both regulators (11, 47, 49). It is likely that the two proteins, which share 67% similarity on the amino acid level (18), recognize similar binding sites, since we observed using EMSAs (data not shown) that purified lactococcal H6-CodY was capable of binding to the upstream region of *B. subtilis comK*, which is a known, direct member of the *B. subtilis* CodY regulon (46). Part of the present study was aimed at gaining insight into the sequence requirements for *L. lactis* CodY recognition of its target promoters. Random and site-directed mutagenesis revealed that a region required for recognition of a promoter area by the CodY repressor, at least in the case of P*oppD*, contains an inversely repeated nucleotide sequence. Therefore, it would be tempting to speculate that this 7-bp IR of the nucleotides ATGTTCA is needed for CodY binding, since regions of dyad symmetry often serve as operator sites for transcriptional regulators.

A sequence comparison of the upstream regions of all known CodY-regulated promoters in *L. lactis* revealed that a subset of these (e.g., *pepC*, *pepN*, *araT*, and *prtPM)* contain an area of dyad symmetry close to their (predicted) promoters. However, these repeats do not seem to have any obvious mutual sequence similarity, and therefore it is possible that they do not serve a role in CodY-mediated regulation. As postulated (47), it could also be that CodY does not recognize a specific nucleotide stretch but that, rather, a topological structure (e.g., bent DNA) in its targets is required for binding.

Further mutational analysis would be of great importance in gaining a better understanding of the sequence requirements for CodY binding. In addition, DNA microarray experiments are currently being performed in order to identify possible new members of the lactococcal CodY regulon, which would provide more *cis* sequence information as well.

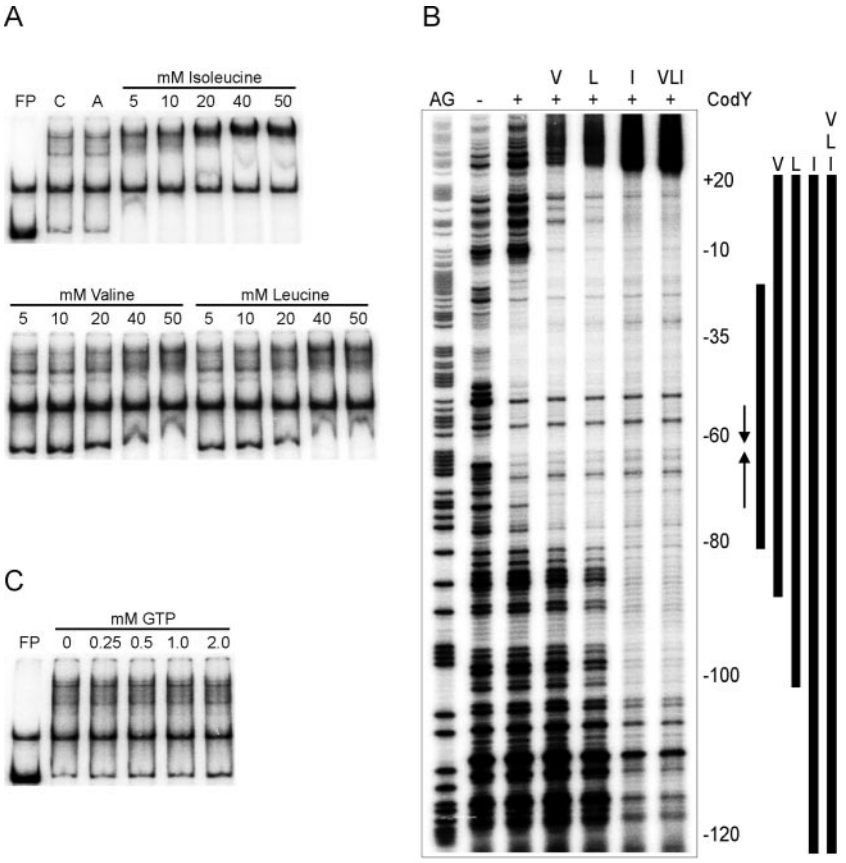

FIG. 6. EMSA and DNase I footprinting analysis of the effects of BCAAs and GTP on H6-CodY binding to P*oppD*. EMSA reactions were carried out as described in the legend to Fig. 2B in the presence of probe *opp162* and 120 nM H6-CodY and with varying concentrations of (A) BCAAs or (C) GTP. All reactions contain H6-CodY except lanes FP, which contain free probe. Lane A contains 50 mM Ala. (B) Footprinting reactions were carried out as described in the legend to Fig. 3 in the absence (−) or presence (+) of 120 nM H6-CodY and 20 mM Val, Leu, Ile, or a combination of these three (lanes V, L, I, and VLI, respectively). A Maxam and Gilbert A+G sequence ladder is present on the left (AG). Numbers on the right indicate base pair positions relative to the TSS. Protected regions are indicated by bars. The vertical arrows indicate the region of dyad symmetry closest to the −35 sequence (see Fig. 1B).

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